RE-EXAMINATION OF THE PRODUCTION OF CYCLOPIA IN FUNDULUS HETEROCLITUS WITH MAGNESIUM CHLORIDE AND ETHYL ALCOHOL[1]

K. T. ROGERS[2]

Biological Laboratories, Harvard University, Cambridge 38, Mass., Marine Biological Laboratory, Woods Hole, Mass. and Department of Zoology, Oberlin College, Oberlin, Ohio

During three seasons (1948, 1949, 1955) attempts were made to produce perfect cyclopean fish for anatomical and physiological studies. Because of commonly accepted, misleading statements in the literature, much time was wasted at first. In brief, the large percentages of cyclopean embryos reported after $MgCl_2$ treatment by Stockard (1907, 1909) are not readily obtainable by this method; but instead, very large numbers of experimental animals must be handled to secure even a few hatched, perfect cyclopean fish.

The first purpose of this paper is to present quantitative data on this negative aspect of the problem. Such data have been lacking in previous reports, although McClendon (1912) has pointed out that the high percentages obtained by Stockard were not obtained by him. A second, positive purpose is to suggest that specific temperature in combination with the chemical treatment is of prime importance. Third, since ethyl alcohol treatment gives a large percentage of cyclopean embryos (although they are extremely unlikely to hatch), precise data on the effective concentrations are presented.

Magnesium Chloride

In work with this salt, gram molecular stock solutions of $MgCl_2 \cdot 6\ H_2O$ were made at the start of each experiment. In the manner of Stockard, 18 parts of this solution were added to 42 parts of sea water, 19 parts to 41 parts of sea water, etc. These were designated by Stockard as 18/60 *M*, 19/60 *M*, etc. The fact that they do not truly bear this relation to a molar solution is not especially significant. If a series of concentrations from completely ineffective to completely lethal is used, the optimum concentration will be included. The method is convenient and allows close comparison with Stockard's work.

In 1948 the experiments used fish obtained May 21st from a live-bait dealer in Boston, and sea water was obtained off the coast away from the city and stored in glass carboys. A liter of stock solution was made with 203.33 grams of $MgCl_2 \cdot 6\ H_2O$ in distilled water. About half an inch of properly diluted solution was added to each batch of eggs in 4″ finger bowls from 3 to 18 minutes after insemination. The bowls were stacked to prevent undue evaporation and stored in

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[2] Present address: Department of Zoology, Oberlin College, Oberlin, Ohio.

TABLE I

1948 $MgCl_2$ experiments

Solution	Dead or undeveloped	Two-eyed embryos	Cyclopean embryos	Double embryos
18/60 $MgCl_2$ (2 bowls)	675	7	0	0
19/60 $MgCl_2$ (8 bowls)	1081	261	2	1
20/60 $MgCl_2$ (2 bowls)	338	31	0	0
Control (sea water)	62	0	0	0

an aquarium room with a temperature range of 16 to 21° C. Solutions were replaced every 48 hours. Undeveloped but dead eggs were counted and removed after the first two days. After 12 days the embryos were preserved and inspected under the compound microscope. The results are presented in Table I.

In 1949 eggs were fertilized at Woods Hole from June 16th to July 5th. The season was early and it became increasingly difficult to find fish which had not already shed. At first fish came from the waters around Woods Hole, but during the last week some were obtained from Barnstable on the north side of Cape Cod.

Sea water from the outlets on the water tables in the laboratory was added to a depth of half an inch, five to ten minutes after fertilization, and replaced by magnesium chloride solutions when the embryos were in the four- to eight-cell stage. Bowls were tipped up and drained as dry as possible in changing solutions, to avoid

TABLE II

1949 $MgCl_2$ experiments

Solution	Died during development	Normal two-eyed embryos	Cyclopean embryos
A. Stock solution made up in sea water			
15/60 $MgCl_2$	3 (12%)	23	0
18/60 $MgCl_2$	21 (36%)	37	0
22/60 $MgCl_2$	35 (81%)	8	0
B. Stock solution made up in distilled water			
15/60 $MgCl_2$	7 (18%)	33	0
16/60 $MgCl_2$	8 (18%)	37	0
18/60 $MgCl_2$	4 (7%)	51	0
19/60 $MgCl_2$	30 (26%)	84	0
20/60 $MgCl_2$	42 (41%)	60	0
21/60 $MgCl_2$	70 (77%)	21	0
23/60 $MgCl_2$	76 (77%)	23	0
25/60 $MgCl_2$	51 (100%)	0	0
C. Sea water controls (adults for second control came from closed brackish pond)			
	2 (6%)	30	0
	52 (48%)	56	0

further dilution. The solutions were replaced every 12 hours; embryos were kept in the solutions for 48 to 72 hours, until the condition of their eyes was clear from inspection under the compound microscope. In contrast to the previous experiments, unfertilized eggs were removed within several hours of insemination; therefore only eggs which passed through the initial cleavage stages were tabulated. Finger bowls were stacked on a desk, and water temperature in the bowls was recorded at times scattered through the day and night. Of 50 recorded temperatures, 28 fell between 23° and 25° C. Only two were below 22° C. (20.5° and 21.0°) and four above 26° C. (26.25°, 26.5°, 27.25°, and 27.5°).

The effect of magnesium chloride dissolved in sea water alone (Stockard, 1907) was tested; 203.33 grams of $MgCl_2 \cdot 6\ H_2O$ were dissolved in one liter of sea water.

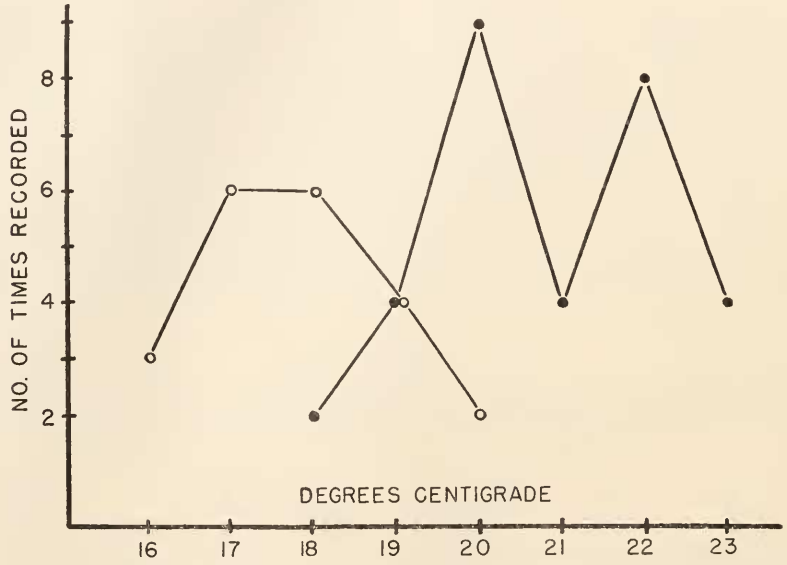

FIGURE 1. Recorded experimental dish temperatures, 1955. Temperature was recorded usually four times a day, and diurnal fluctuation in the laboratory room was kept as low as possible. Open circles represent recordings before June 12th, solid circles represent recordings after June 12th.

This is not a molar solution but the salts in sea water would cause inexactness anyway. Closer measurement seemed unnecessary since a range of concentrations was to be employed. This stock solution was then added to sea water in the same manner as the distilled water solutions of magnesium chloride had been. In another series, a gram molecular solution of $MgCl_2 \cdot 6\ H_2O$ in distilled water was titrated colorimetrically. This solution was added to sea water and the eggs and bowls were handled as described. The results of these two series are shown in Table II.

In 1949 because of the failure of the magnesium chloride treatment to produce any cyclopean embryos, more extensive series were not run. Alcohol treatment instead was used to secure cyclopeans for anatomical study (Rogers, 1952). The alcohol method had the disadvantage of not allowing embryos to develop well enough to hatch when used in concentrations that would produce cyclopia. Ac-

cordingly, when free-swimming cyclopean fish were desired for physiological tests in 1955, very large series of $MgCl_2$ experiments were carried out. From the results of 1948 and 1949 there was a suggestion that temperature might have an effect. The hurricane-flood damage of the previous season at Woods Hole prevented use of the cold rooms for some time in 1955, so experiments were carried out on a laboratory table at first. Room temperature was kept as constant as diurnal fluctuation allowed and experimental dish temperature was recorded at times scattered through the day and night. These experiments fall into two groups: those in which the embryos passed the critical eye-determining period during cold weather between June 5th and 11th (series A), and those after a storm temporarily

TABLE III

1955 $MgCl_2$ experiments

No. of bowls	Solution	Died during development	Normal two-eyed embryos	Cyclopean embryos	Other abnormals	% of living that were abnormal
		Series A				
3	15/60 $MgCl_2$	54—(31%)	120	0	0	
19	18/60 $MgCl_2$	682—(28%)	1713	38	22	3.4%
23	19/60 $MgCl_2$	864—(28%)	2139	58	60	5.2%
11	20/60 $MgCl_2$	1052—(51%)	967	17	38	5.4%
2	21/60 $MgCl_2$	136—(52%)	124	2(?)	1	
3	24/60 $MgCl_2$	514—(95%)	26	1(?)	1	
1	25/60 $MgCl_2$	153—(88%)	20	0	0	
2	Sea water controls	46—(10%)	395	0	0	
		Series B				
27	19/60 $MgCl_2$	Not recorded	3488	79	15	2.6%
		Series C				
	19/60 $MgCl_2$					
24	10° C.	1429—(100%)	0	0	0	
24	18° C.	292—(20%)	1070	42	26	6.0%
24	25° C.	137—(11%)	1156	3	7	0.9%

prevented obtaining adults, or the warmer period between June 12th and 19th (series B). The recorded temperatures during these two periods are plotted in Figure 1.

A third series was run in temperature-controlled chambers from June 22nd to July 3rd (series C). The 10° cold room varied from 6° to 10° C., but was between 9.5° and 10° C. most of the time. The 18° room varied between 16.5° and 19.1° C.; the 25° chamber, between 25° and 26° C. The two colder chambers were sizeable rooms, whereas the 25° chamber was the size of a household refrigerator. Twenty-four bowls were kept at each temperature. In 16 cases batches of eggs, each from single pair matings, were divided and a third of each batch placed in a

bowl at each temperature, to have a control on hereditary disposition as far as possible. The remaining eight bowls at each temperature had to be made up by dividing into thirds, batches of eggs combined from stripping many adults. After the eyes were determined, embryos were returned to sea water and kept at laboratory room temperatures (around 24° C. at that time).

Methods of handling all the bowls followed those described for 1949. A gram molecular solution of $MgCl_2 \cdot 6\ H_2O$ in distilled water was used in every case as the stock solution; no sea water stock solutions of $MgCl_2$ were employed. All of the 16,987 eggs were inspected under the dissecting microscope at least twice, once in the cleavage stages, when additional, non-developing eggs were removed, and again to classify the embryos after the eyes had developed. In addition, all 410 abnormal embryos were kept in sea water for a further period so that they could be more certainly classified after further development, although even then reasonable doubt sometimes remained. Complete records were kept on every bowl; the salient features of the 64 pages of data are summarized in Table III.

Alcohol

Stockard (1910) prepared mixtures on a percentage basis and found that from 3 to 9% ethyl alcohol in sea water produced viable cyclopean embryos, although he stated (p. 369) that "only a few of the alcoholic specimens ever develop sufficiently to hatch and swim about as do the Mg embryos." In his experiments batches of 60 to 100 eggs were exposed to 60 cc. of solution "for (not) more than twenty-four to thirty-six hours" following the eight-cell stage and then returned to sea water. Mention was not made of the methods employed, if any, to prevent evaporation.

In the present work tests were made to determine what precautions were necessary for the prevention of rapid evaporation. At 20° C. the partial pressure of ethyl alcohol in a 10% solution in water (by weight) is 28% of the total vapor pressure of the solution and at 40° C. is 34% (International Critical Tables). Initially comprising only 10% of the solution, the alcohol will contribute almost a third of the initial evaporation. The percentage of alcohol in the solution will fall rapidly if evaporation is allowed to take place. The salts in sea water will have no significant effect on the situation (personal communication from Professor G. B. Kistiakowsky). Stacking of finger bowls seemed inadequate to prevent evaporation of this magnitude and sealing the bowls would be time-consuming; a glass plate larger than the finger bowl was therefore laid over the aperture of each bowl. As a test of the effectiveness, covered and uncovered bowls were left 17 hours at 20.5° C. in a laboratory room. Each initially contained 55 cc. of a 10% (by volume) solution of absolute ethyl alcohol in sea water. At the end of the period the covered bowl contained 54.75 cc. (the difference from the initial volume being within the limits of error of measurement); the uncovered bowl contained only 48 cc. Since in the experiments solutions were replaced every 12 to 17 hours, and the dishes were covered, evaporation was not significant.

The experiments with alcohol were carried out in much the same way as those with $MgCl_2$. Unripe and unfertilized eggs were removed at the start of the experiment so that only eggs that were initially developing were tabulated. The embryos were returned to sea water in the 1949 series after 24½ to 33½ hours, and in the 1955 series after 51 to 53½ hours of exposure to the alcohol mixtures. In the

latter case the embryos were left in the alcohol solutions until they could be seen to be developing eyes in the less lethal concentrations. Experiments in 1949 were at temperatures most frequently between 23° and 25° C. as noted for the $MgCl_2$ experiments, and in 1955 entirely between 21.5° and 22.8° C. Lethality of the solutions is summarized in Table IV.

It should be noted that the two embryos counted as having survived the 5% alcohol treatment in reality failed to axiate. In these two cases the yolk spheres did not disintegrate and unorganized masses of cells with a few chromatophores developed upon their surfaces. The 5% alcohol mixture thus for all practical purposes was lethal. This held true when it was used for 32 hours or for 51½ hours (the two surviving eggs were in the latter group).

The 2% and 2½% solutions (1,268 embryos) produced only about a dozen perfect cyclopeans, which did not hatch. A dozen embryos in these bowls did hatch, including one synophthalmic embryo. The 3% solution proved best for production of cyclopia while still allowing moderately good development of body form. In the 1949 series with 3% alcohol, embryos were tabulated individually,

TABLE IV

1949 and 1955 alcohol experiments

No. of bowls	% of alcohol	Total embryos	% embryos dead before eyes developed
4	2%	650	5%
3	2½	618	4
9	3	692	29
4	4	697	73
3	4½	608	89
4	5	869	99.8
		4,134	

the living embryos giving the following totals: 128 cyclopeans including some with a single optic cup but two lenses, 43 monophthalmics, 60 two-eyed embryos including some synophthalmics which had two optic cups, each with lens and pupil, and 26 individuals that could not be classified. None of the embryos treated with 3% alcohol hatched. The 4% solution yielded an even higher percentage of cyclopean embryos among the survivors, but general body development was so inhibited and so many of the eyes were greatly reduced in size that results did not compare favorably with those at 3% concentration. The 4½% solution allowed only abortive attempts at axiation, and as noted above, the 5% solution was lethal.

DISCUSSION

The first point that is demonstrated by the present data is that the $MgCl_2$ method will not ordinarily give large percentages of cyclopean embryos. McClendon (1912) stated (p. 139), "In numerous experiments covering an entire season, I failed to obtain as high a per cent of cyclopia with magnesium chloride as recorded by Stockard." Stockard (1909) admitted difficulties in repeating his

initial experiment. Yet McClendon's work has largely been overlooked because of its negative character. Stockard classified all grades of synophthalmia as cyclopia, and yet the percentages of *all* abnormals in the best instances in the present experiments remain at about one-tenth of the fifty per cent cyclopia reported by Stockard. In Table III the numbers of cyclopean embryos probably are too high, since whenever there was doubt in cases where the eye seemed to be cyclopean but was so reduced as to make it difficult to be certain, the embryo was classified "cyclopean" rather than "other abnormal." The totals for *all* abnormals, however, are accurate.

The chances of obtaining free swimming cyclopean fish are not as good as the tabulated figures would make them appear to be. Only three cyclopean and four synophthalmic embryos hatched in the 1955 experiments. In addition, 21 monophthalmics, 17 with one eye partially reduced, and three anophthalmics hatched. All of these came from Series A except for three monophthalmics and two with one eye partially reduced from Series B, and one synophthalmic and two monophthalmics from untabulated bowls started after the temperature-controlled experiments and upon which individual records were not kept. No abnormals from the temperature-controlled experiments (Series C) hatched, a fact for which no explanation is apparent.

The influence of temperature in the $MgCl_2$ work now seems clear. Loeb (1915) and Kellicott (1916) reported some normally developing Fundulus embryos after eggs had been exposed in the early stages for a time to 7° C., and 8 to 10° C., respectively. Many embryos died or were markedly abnormal after such exposure, however. In the present work it is clear that the 19/60 $MgCl_2$ mixture couples its effect with that of cold in an additive way, for not one embryo survived of 1,429 exposed to 10° C. in the $MgCl_2$ solution (Series C, Table III). Most of them reached the late blastula stage before disintegrating. At temperatures for the most part of 17 to 18° C., between 5 and 6% of 3,395 surviving embryos in 19/60 $MgCl_2$ were abnormal (Series A and C). At temperatures for the most part of 20 to 22° C., 2.6% of 3,582 surviving embryos in 19/60 $MgCl_2$ were abnormal (Series B). Finally at temperatures between 25 and 26° C. only 0.9% of 1,166 surviving embryos in 19/60 $MgCl_2$ were abnormal (Series C). Unfortunately there were no means to test temperatures between 11 and 16° C. There remains the possibility that the optimum temperature for obtaining cyclopean embryos in the $MgCl_2$ solutions lies within this range.

In addition to the temperature differences there is the possibility that differences in hereditary susceptibility may cause variation in results. The incidence of any abnormality was so low in the 16 bowls kept at 25° C., for which there were control bowls kept at 18° C., that no significant results were obtained. Nevertheless, one cannot fail to be impressed with the great variation that does occur among batches of eggs handled in exactly the same way under the same conditions. The 19/60 $MgCl_2$ tests in Series A, Table III, are a good example. Of 58 cyclopean embryos, 46 occurred in three bowls, and of the total of 23 bowls, 14 contained no cyclopeans at all.

The results of the alcohol work indicate that Stockard did not control evaporation to any extent since he found 3% to 9% alcohol to be effective in producing living cyclopean embryos, and the present work shows 5% alcohol to be lethal if that concentration is maintained. Desired results may be obtained in percentages

of alcohol steadily decreasing with time, but conditions cannot be repeated without control of humidity, temperature, and air currents.

Summary

1. Under the conditions of the present $MgCl_2$ experiments, which followed those of Stockard as closely as possible except for temperature control, 2.0% of 11,912 surviving embryos were cyclopean; 0.3% were perfect cyclopean with an eye close to normal size, of which only three individuals hatched.

2. The optimum concentrations of $MgCl_2$ at the temperatures of these experiments were those which Stockard designated 18/60, 19/60, and 20/60 *M*.

3. With 19/60 *M* $MgCl_2$ solution, 10° C. proved lethal, while 17 to 18° C. was the optimum temperature used, with effectiveness falling as the temperature increased to 26° C. It was not possible to test temperatures of 11 to 16° C.

4. At temperatures of 21.5 to 25° C., 3% alcohol in sea water was the most effective in producing cyclopean embryos with moderately well developed body form; 4½% alcohol allowed only abortive axiation and 5% alcohol was lethal.

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